The (p)ppGpp synthetase RelA contributes to stress adaptation and virulence in *Enterococcus faecalis* V583

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Guanosine penta- and tetraphosphate [(p)ppGpp] are two unusual nucleotides implied in the bacterial stringent response. In many pathogenic bacteria, mutants unable to synthesize these molecules lose their virulence. In Gram-positive bacteria such as *Enterococcus faecalis*, the synthesis and degradation of (p)ppGpp mainly depend on the activity of a bifunctional enzyme, encoded by the *relA* gene. By analysing $\Delta relA$ and $\Delta relQ$ (which encodes a protein harbouring a ppGpp synthetase activity) deletion mutants, we showed that RelA is by far the main system leading to (p)ppGpp production under our experimental conditions, and during the development of a stringent response induced by mupirocin. We also constructed a mutant ($\Delta relA_{sp}$) in which a small part of the *relA* gene (about 0.7 kbp) encoding the carboxy-terminal domain of the RelA protein was deleted. Both *relA* mutants were more resistant than the wild-type strain to 0.3 % bile salts, 25 % ethanol and acid (pH 2.3) challenges. Interestingly, the $\Delta relA_{sp}$ mutant grew better than the two other strains in the presence of 1 mM H₂O₂, but did not display increased tolerance when subjected to lethal doses of H₂O₂ (45 mM). By contrast, the $\Delta relA$ mutant was highly sensitive to 45 mM H₂O₂ and displayed reduced growth in a medium containing 1 M NaCl. The two mutants also displayed contrasting virulence phenotypes towards larvae of the Greater Wax Moth infection model *Galleria mellonella*. Indeed, although the $\Delta relA$ mutant did not display any phenotype, the $\Delta relA_{sp}$ mutant was more virulent than the wild-type strain. This virulent phenotype should stem from its increased ability to proliferate under oxidative environments.

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INTRODUCTION

Enterococcus faecalis belongs to the lactic acid bacteria, and is a natural member of the human intestinal microflora. This bacterium is used in several food fermentations (Giraffa, 2003; Hugas *et al.*, 2003) and as a probiotic (Mercenier *et al.*, 2003). However, it is also an important opportunistic pathogen, and represents one of the main causes of nosocomial infections of the bloodstream, surgical wounds, heart valves and urinary tract in USA and Europe (Noskin *et al.*, 1991; Schaberg *et al.*, 1991). One of the most notable physiological traits of *E. faecalis* is its exceptional resistance to harsh environments, contributing to its persistence in hospitals despite decontamination procedures. For example, *E. faecalis* is able to survive

in 6.5 % NaCl, at pH values between 4.0 and 9.6, and at growth temperatures from 10 to 45 °C (Sherman, 1937). These physiological characteristics are obviously important for its use as a probiotic or as a starter culture, but also contribute to its survival in diverse commensal and pathogenic environments.

The stringent response allows bacteria to adapt to nutrient starvation and other environmental stresses. During the stringent response, intracellular signals, including guanosine-3'-diphosphate-5'-triphosphate (pppGpp) and guanosine-3',5'-bisphosphate (ppGpp) [collectively referred to as (p)ppGpp], are synthesized by phosphorylation of GDP and GTP to ppGpp and pppGpp, respectively, using ATP as a phosphate donor. In *Escherichia coli*, two proteins are involved in (p)ppGpp synthesis: RelA, which generally synthesizes (p)ppGpp in response to amino acid limitation; and SpoT, which also displays a (p)ppGpp hydrolase activity, and is responsive to changes in carbon, phosphate, and fatty acid availability as well as to changes in temperature and

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Abbreviation: RT qPCR, real-time quantitative PCR.

osmolarity (Cashel *et al.*, 1996; Murray & Bremer, 1996; Wendrich *et al.*, 2002). Most Gram-positive bacteria have a single RelA protein which exhibits both (p)ppGpp synthetase and hydrolase activities (Mechold *et al.*, 1996; Wendrich & Marahiel, 1997; Mittenhuber, 2001).

The stringent response was first described as an adaptation to amino acid starvation. However, (p)ppGpp accumulation also influences many other physiological functions, including competence (Inaoka & Ochi, 2002), morphological differentiation and production of antibiotics (Sun *et al.*, 2001; Jin *et al.*, 2004a, b), thermotolerance (Yang & Ishiguro, 2003), adaptation to oxidative stress (Mostertz *et al.*, 2004), osmotic stress (Okada *et al.*, 2002) and sensitivity to antibiotics (Greenway & England, 1999). In the lactic acid bacterium *Lactococcus lactis*, *relA** mutants producing a C-terminal-truncated RelA protein, display an increased resistance to multiple stresses, suggesting a major role for (p)ppGpp in lactococcal stress adaptation (Rallu *et al.*, 2000).

Among pathogens, *relA* null mutants exhibit a large decrease in virulence, as has been observed for *Salmonella enterica* serovar Typhimurium (Pizarro-Cerda & Tedin, 2004), *Vibrio cholerae* (Haralalka *et al.*, 2003), *Mycobacterium tuberculosis* (Dahl *et al.*, 2003) and *Listeria monocytogenes* (Taylor *et al.*, 2002).

To determine what role the (p)ppGpp synthetase RelA plays in an organism that possesses both commensal and pathogen properties, we constructed two *relA* deletion

mutants of *E. faecalis*, resulting in (i) the total absence of RelA and (ii) a C-terminal-truncated RelA protein. We report the characterization of these mutants, from their (p)ppGpp production to their ability to adapt to stress, and assess the changes in virulence in a *Galleria mellonella* infection model.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. We used as the parental *E. faecalis* strain an erythromycin-sensitive derivative of the clinical isolate *E. faecalis* V583 (Sahm *et al.*, 1989), which was generated in our laboratory by A. Benachour. This derivative is hereafter referred to simply as strain V583. Unless otherwise indicated, *E. faecalis* V583 and its derivatives were grown at 37 °C with shaking in M17 medium supplemented with 0.5% glucose (GM17) or without shaking in a chemically defined medium (DM) adapted from Pichereau *et al.* (1999). When necessary, erythromycin (150 µg ml⁻¹) was added. *Escherichia coli* Top10 (Invitrogen) was cultured with shaking at 37 °C in LB medium with ampicillin (100 µg ml⁻¹) when required.

Adaptation and challenge conditions. Cultures were grown to an OD₆₀₀ of 0.5 in GM17 and were harvested by centrifugation (4500 r.p.m., 15 min). Cells were washed once and either transferred into DM for 2 h or directly used for challenge in GM17. Bacterial cells were then pelleted by centrifugation (4500 r.p.m., 15 min) and challenged with 0.3% (w/v) bile salts (50% sodium cholate/50% sodium deoxycholate; Sigma Aldrich) for 30 s. Viable counts were determined by spreading 1 ml dilutions onto GM17 agar plates

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristic(s)	Reference or source
<i>Enterococcus faecalis</i>		
V583 Ery ^S *	Erythromycin-sensitive derivative of <i>E. faecalis</i> V583; wild-type strain	A. Benachour
Δ relA	V583 isogenic derivative; <i>ef1974</i> complete deletion mutant	This study
Δ relAsp	V583 isogenic derivative; <i>ef1974</i> partial deletion mutant	This study
Δ relAcomp	Δ relA strain complemented by allelic exchange	This study
Δ relQ	V583 isogenic derivative; <i>ef2671</i> complete deletion mutant	This study
Δ relA- Δ relQ	V583 isogenic derivative; <i>ef1974</i> and <i>ef2671</i> complete deletions mutant	This study
<i>Lactococcus lactis</i>		
IL1403	Wild-type strain	Chopin <i>et al.</i> (1984)
MG1363	Wild-type strain	Gasson (1983)
VI6963	MG1363 derivative <i>relA::ISS1</i>	Rallu <i>et al.</i> (2000)
<i>Escherichia coli</i>		
Top10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> <i>galU</i> <i>galK</i> Δ (<i>ara-leu</i>)7697 <i>rpsL</i> (Str ^R) <i>endA1</i> <i>nupG</i>	Invitrogen
Plasmids		
pGEM-T	Amp ^R <i>lacZ'</i>	Promega
pGEM-T-A5A6	pGEM-T carrying A5-A6 amplification (4.1 kbp)	This study
pGEM-T-Asp4A6	pGEM-T carrying Asp4-A6 amplification (2.2 kbp)	This study
pGEM-T-ef2671	pGEM-T carrying 2671,1SalI-2BglII amplification (2.5 kbp)	This study
pMAD	<i>ori</i> pE194 ^{ts} ; Ery ^R Amp ^R <i>bgaB</i>	Arnaud <i>et al.</i> (2004)
pMAD- Δ A3A4	pMAD carrying the whole <i>ef1974</i> gene deletion (2.2 kbp)	This study
pMAD- Δ Asp5A4	pMAD carrying the 0.7 kbp deletion of <i>ef1974</i>	This study
pMAD- Δ ef2671	pMAD carrying the 0.6 kbp deletion of <i>ef2671</i>	This study

*Referred to as strain V583 throughout paper.

(Difco), followed by incubation at 37 °C for 48 h. Three independent experiments were performed and duplicate platings were carried out for each point. A similar protocol was used to test other challenge conditions (45 mM H₂O₂, 25%, v/v, ethanol, pH 2.3, 60 °C), except that exposure was for 30 min in these experiments. Means of at least three independent experiments, with less than 10% variation, are shown.

Analysis of (p)ppGpp levels. Cells were grown to an OD₆₀₀ of 0.5 at 37 °C with shaking in GM17 and harvested by centrifugation. Cells were washed once and resuspended in DM or in GM17, with 50 µg mupirocin ml⁻¹, 0.75 M NaCl or 0.75 mM H₂O₂ added later. Then cultures were incubated without shaking for 2 h in the presence of 150 µCi (5.55 MBq) ³²P-labelled H₃PO₄ ml⁻¹. Samples were pelleted and washed with 200 µl fresh medium and resuspended in 25 µl cold ultrapure H₂O. After addition of an equal volume of 13 M formic acid, samples were vigorously shaken and frozen at -20 °C for one night, subjected to three freezing/thawing cycles, and centrifuged for 5 min to pellet cell debris. A 20 µl aliquot of the supernatants was spotted onto polyethyleneimine (PEI)-cellulose plates (Selecto Scientific) for separation by TLC in 1.5 M KH₂PO₄ (pH 3.4). Labelled nucleotides were visualized following chromatography by electronic autoradiography and quantified by densitometry with a

Cyclone PhosphorImager (Packard Instrument Company) and its associated software (Optiquant). The identities of the labelled pppGpp and ppGpp were deduced from their positions in the chromatogram relative to the migration of radioactive ATP (by performing co-migration experiments) and by comparison with published results (Kasai *et al.*, 2004).

Real-time (RT) quantitative PCR (qPCR) RT qPCR was performed exactly as described by Giard *et al.* (2006). Specific primers were designed using Primer3 software (<http://frodo.wi.mit.edu/primer3/>) (Table 2) with the following parameters: amplicon length, 99–101 bp; primer length, 19–21 nt; primer melting temperature, 59–61 °C. Total RNA of different strains treated with DNase I (GE Healthcare) and the DNA-free kit (Ambion) were reverse-transcribed using the Omniscript enzyme (Qiagen) and random hexamer primers, according to the manufacturer's recommendations. RT qPCR was performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen). Quantification of 23S rRNA transcript levels was used as an internal control. All the experiments were performed twice and in duplicate with two different RNA samples, using the Bio-Rad iCycler iQ detection system. The value used for the comparison of gene expression in various strains was the number of PCR cycles required

Table 2. Oligonucleotide primers used in this study

Primer	Oligonucleotide sequence (5'–3')*
Mutagenesis	
UP	GTAAAACGACGGCCAGT
RP	CAGGAAACAGCTATGAC
A1	GGCGCATGCTAAAAACAAGACGGTAC (<i>SphI</i>)
A3	CAATTTTGATGACCGCTGG
A4	GTGTACCAGATGTTTATAG
A5	AATGGCATGGTATTTTCATGAAAGAAG
A6	TCGGAGATCTGATTTCTTGGTATTG (<i>BglII</i>)
Asp4	AATTCATGGGAATTAGAAGATACTG (<i>NcoI</i>)
Asp5	TTATTAATCATGCCCTTTAATAATG
A3bis	GACTCATATGACACAGTCTTGG
A4bis	CCTTCGTAGATTTTTAATATTATC
2671,1SalI	GCAAGTCGACTTTCTTCTGTGCTTAACAGAC (<i>SalI</i>)
2671,2BglII	CCTGAGATCTCACCTTTGCTAATAACCC (<i>BglII</i>)
2671,3BamHI	AAACGGATCCCACTCTCGTTCCATGG (<i>BamHI</i>)
2671,4BamHI	CCGTGGATCCCTAGAAAACGAATAC (<i>BamHI</i>)
2671,3bis	TTCCGTCGACAAAAGTGGTCTTCTCTGTG (<i>SalI</i>)
2671,4bis	ATTTGGATCCGCTCTAATAGTGTGTC (<i>BamHI</i>)
RT qPCR	
23S-L	GTTCCCTCAGAATGGTTGGA
23S-R	CACTAAGCCCGACTTTCGTC
1972L	TGAAAGACATTCTGGAGATGGA
1972R	CACGGTACCACCTAGTTTTGAA
1973L	AGGCAATCGTCCAGTTTTA
1973R	ACCGCTATTCTTCTGCTTCT
1974L	TGATGTTGTCGAAGATACGGA
1974R	CCTAGTTTCGTCACGCCATC
1975L	TGTCAAATGGGACCACAAAA
1975R	CTTGGTGTCTGCTGTGATG
1976L	TGGCGTTTGCTAAAAACAAGA
1976R	CCACAAAACCGACTTTCGTT
2671L	TGTGGAAGAACGGGACTACA
2671R	TTTTCCCCGGTGACTAATTG

*Underlined bases correspond to the restriction sites.

to reach the threshold cycle (C_T). To relate the C_T value to the abundance of an mRNA species, C_T was converted to 'n-fold difference' by comparing mRNA abundance in the V583 wild-type strain to that obtained with the *relA* mutant strains. The n-fold difference was calculated by the formula ($n=2^{-x}$) when the $C_{T \text{ mutant}} < C_{T \text{ V583}}$ and ($n=2^x$) when $C_{T \text{ mutant}} > C_{T \text{ V583}}$, with $x = (C_{T \text{ mutant}} - C_{T \text{ V583}})$.

Sample preparation for microarray analysis. *E. faecalis* strains V583, $\Delta relA$ and $\Delta relAsp$ were cultured in GM17 to mid-exponential phase. RNAs were extracted using the RNeasy midi prep kit (Qiagen). RNA samples were subjected to two DNase treatments, using successively DNase I (GE Healthcare) and the DNA-free kit (Ambion). DNA degradation was verified by PCR amplification.

cDNA preparation, fragmentation, labelling and hybridization were performed as described in the Affymetrix manual (*GeneChip Expression Analysis Technical Manual: Prokaryotic Target Preparation*; P/N 702232 Rev 2). Washing and scanning were performed using a GeneChip Fluidics Station 450. The arrays were read at 570 nm with a resolution of 1.56 μm using an Affymetrix GeneChip Scanner 3000 7G. Results were analysed and compared using GeneChip Operating Software (GCOS) version 1.4.

Construction of *E. faecalis* mutants and functional complementation. To obtain the $\Delta relA$ mutant, a DNA fragment of 4.1 kbp (or 2.6 kbp) was amplified using DNA primers A5 and A6 with flanking restriction sites as described in Table 2. Both PCR products were purified and ligated into pGEM-T. Ligated products were transformed into electrocompetent cells of *Escherichia coli* Top10. The resulting plasmid was purified and subjected to PCR amplification using primers A3 and A4, located 1 kbp inside the cloned fragment. The resulting PCR product, consisting of the whole pGEM-T vector flanked by the two 1 kbp extremities of the DNA region initially cloned, was then self-ligated, yielding constructions carrying the *relA* deletion. Exactly the same strategy was used to create the constructions containing the *relAsp* deletion (i.e. deletion of 0.7 kbp in the 3' extremity of $\Delta relAsp$) and the *ef2671* deletion ($\Delta relQ$), but using the corresponding primers described in Table 2.

After cloning, the engineered *rel* and *ef2671* derivatives, as well as the whole A5–A6 fragment (for functional complementation experiments), were amplified using primers UP and RP, digested using the appropriate restriction enzymes (i.e. *EcoRI* and *BglII* for $\Delta relA$, and *NcoI* and *BglII* for $\Delta relAsp$), cloned into the thermosensitive plasmid pMAD (Arnaud *et al.*, 2004), and finally transformed into electrocompetent cells of *Escherichia coli* Top10. The desired structures of recombinant plasmids (pMAD- $\Delta A3A4$, pMAD- $\Delta Asp5A4$, pMAD- $\Delta A5A6$ and pMAD- $\Delta 2671$), were verified by restriction analysis and PCR amplification. These plasmids were subsequently used to transform *E. faecalis* V583. Gene replacement was accomplished via double crossover using a method based on the conditional replication of the pMAD plasmid as described by Arnaud *et al.* (2004). Briefly, after electroporation, cell suspensions were plated onto GM17 agar containing 150 μg erythromycin ml^{-1} and 80 μg X-Gal ml^{-1} . After 48 h incubation at 30 °C, the resulting erythromycin-resistant blue colonies were selected and grown twice to exponential phase at 42 °C in the presence of erythromycin. To induce excision of the recombinant plasmid vector, cultures were incubated for 4 h at 30 °C, followed by incubation at 42 °C overnight without antibiotics. This step was repeated four or five times. Erythromycin-sensitive white colonies, which indicated that the plasmid had been excised, were selected by plating on GM17 agar containing 80 μg X-Gal ml^{-1} at 37 °C. The gene deletion of the white erythromycin-sensitive colonies was verified by PCR, expression analyses (Fig. 1), and the whole region potentially involved in recombination (from –1132 to +3168, relative to the position of the *ef1974* translational start) in the

chromosomes of all mutants was sequenced, using the dideoxy chain termination kit GenomeLab DTCS (Beckman Coulter).

Virulence in the *Galleria mellonella* model. To test for attenuation of the mutant strains, 100 μl overnight culture grown in GM17 was transferred to 10 ml fresh GM17, and incubated for 24 h at 37 °C. Four millilitres of culture was pelleted and washed twice in 1 ml saline water (0.9%, w/v, NaCl), and resuspended in saline water. For each strain, 15 randomly chosen *Galleria mellonella* caterpillars (about 200 mg body weight) were used, and each experiment was repeated at least three times. A 1 ml Terumo syringe was used to inject 10 μl of washed cells into *G. mellonella* larvae via the penultimate segment near the hindmost right proleg. After injection, the caterpillars were incubated at 37 °C in plastic containers, and the number of surviving caterpillars was scored every 2 h after 15 h of infection. Caterpillars were considered to be dead when they displayed no movement in response to touch. Results represent the means of at least three biological replicates exhibiting less than 15% variation.

RESULTS AND DISCUSSION

The *ef1974* gene is responsible for the production of (p)ppGpp in *E. faecalis* V583

The gene encoding the putative RelA protein was identified in the *E. faecalis* V583 genome (Paulsen *et al.*, 2003). The *E. faecalis relA* gene (*ef1974*) encodes a 737 aa protein that shares high levels of identity to the *Bacillus subtilis* RelA protein (63% identity, 79.6% similarity). The *ef1974* locus is flanked by (i) a 753 bp gene (*ef1975*) encoding a conserved hypothetical protein harbouring an RNA methyltransferase domain, 215 bp upstream of *relA*, and (ii) a 447 bp gene encoding a D-tyrosyl tRNA deacylase (24 bp downstream of *relA*) (Fig. 1a). It is noteworthy that (i) no ρ -independent terminators could be identified in the intergenic regions, and (ii) both genes are transcribed in the same orientation as *ef1974*, suggesting an operon structure.

To determine the role of RelA on stress adaptation and virulence of *E. faecalis*, we constructed two *relA* mutants by allelic replacement using the thermosensitive conditional replication pMAD system (see Methods). The first mutant, called $\Delta relA$ in this study, carried an almost complete deletion of the *ef1974* locus, while the second, named $\Delta relAsp$, carried a partial deletion of the 3' extremity of the gene (Fig. 1a). This mutant was designed so that it corresponded to the *L. lactis* mutant previously created and characterized by Rallu *et al.* (2000); RelAsp consisted of 513 aa, of which the 500 N-terminal ones exactly (ascertained by DNA sequencing) corresponded to those of the wild-type RelA protein, and contained 13 additional amino acids at its C-terminal extremity (QMFIIVFVEPMGKC). Using the same method (i.e. the pMAD system), we also constructed a $\Delta relA$ complemented strain, named $\Delta relAcomp$, in which the wild-type *ef1974* gene was reintroduced into the chromosome of the *relA* deletion mutant, and a complete deletion mutant of the *ef2671* locus ($\Delta relQ$), which encodes a hypothetical protein

Fig. 1. (a) Schematic representation of the *ef1974* locus and its genetic environment in the wild-type strain V583, and the $\Delta relA$ and $\Delta relAsp$ mutants. (b) Microarray hybridizations results for the *ef1974* locus. V583 Affymetrix chips were hybridized with cDNA prepared from RNAs from strains V583, $\Delta relA$ and $\Delta relAsp$. For each strain, fluorescence results obtained for the 14 *ef1974* probes present in the chips are shown. Red, match; green, mismatch; grey, masked.

recently characterized as an alternative (p)ppGpp synthetase in *E. faecalis* OG1RF (Abranches *et al.*, 2009). A $\Delta relA$ - $\Delta relQ$ double deletion mutant was also constructed.

All mutants were verified by PCR amplification using primers flanking the mutated regions, and DNA sequencing of the whole region potentially involved in the recombination process (including the cloned parts of neighbouring genes; data not shown). In addition, it is noteworthy that whole-genome transcriptional profiling using a custom *E. faecalis* Affymetrix chip showed that (i) no *ef1974* transcript was observed in the $\Delta relA$ background, and (ii) the transcription of the neighbouring genes was not significantly modified in the $\Delta relA$ mutant (Table 3). Very similar results were obtained by using RT qPCR

(Table 3), thus showing that the $\Delta relA$ mutations have no polar effect on the transcription of neighbouring genes. In the $\Delta relAsp$ mutant, microarray experiments revealed that the transcription of the *ef1974* locus was reduced 4.1-fold (1.6-fold by RT qPCR). It should be noted that very similar results were obtained with DNA chips hybridized with RNAs from the corresponding strains deprived of plasmid pTEF2 (as annotated in <http://cmr.jcvi.org>), and that the transcription profiles of both mutants did not reveal obvious inactivation (i.e. lack of expression) of any other gene in the genome (data not shown). The fluorescence values measured individually for the 14 antisense probe pairs corresponding to *ef1974* mRNA on the chips showed that (i) only eight probes hybridized with RNAs extracted

Table 3. Comparison of expression of *rel* and neighbouring genes analysed by RT qPCR and microarrays

Locus	Function	RT qPCR		Microarray	
		$\Delta relA/V583$	$\Delta relAsp/V583$	$\Delta relA/V583$	$\Delta relAsp/V583$
<i>ef1972</i>	Hypothetical protein	-1.47 ± 0.01	-2.2 ± 0.13	-1.55	-2.13
<i>ef1973</i>	D-Tyrosyl-tRNA deacylase	-1.3 ± 0.09	-1.77 ± 0.15	1.1	-1.34
<i>ef1974</i>	GTP pyrophosphokinase	-2920 ± 955	-1.61 ± 0.07	-1500	-4.1
<i>ef1975</i>	Hypothetical protein	-1.05 ± 0.12	1.16 ± 0.01	1.22	1.08
<i>ef1976</i>	Ribosomal protein L11 methyltransferase	-1.25 ± 0.04	1.39 ± 0.41	1.04	-1.03
<i>ef2671</i>	Conserved hypothetical protein	-2.24 ± 0.18	-1.28 ± 0.16	-1.11	-1.01

from the $\Delta relA$ mutant, thus confirming the partial deletion of the *relA* gene in this mutant, and (ii) the fluorescence values observed for these eight probes were slightly higher than the corresponding ones in the wild-type sample (Fig. 1b).

To determine whether mutations in the *relA* gene resulted in alteration of the (p)ppGpp pools, *E. faecalis* cultures were fed $150 \mu\text{Ci } ^{32}\text{P-labelled H}_3\text{PO}_4 \text{ ml}^{-1}$ in both GM17 and DM culture media, and the nucleotides were extracted and separated by PEI TLC. Results shown in Fig. 2(a, b) show that in the $\Delta relA$ mutant, ppGpp and pppGpp were undetectable. In the $\Delta relA$ mutant, the relative amount of ppGpp was not significantly affected, as compared to that observed in the wild-type strain, and pppGpp was reduced. As expected, *relA* functional complementation restored the ability of the $\Delta relA$ mutant to produce (p)ppGpp.

We did not observe production of (p)ppGpp by the $\Delta relA$ mutant generated in this study. However the possibility that other sources of (p)ppGpp biosynthesis in *E. faecalis* V583 may be active under other conditions had to be considered. Indeed, it is noteworthy that novel (p)ppGpp synthetases, i.e. YwaC and YjbM in *B. subtilis* (Nanamiya

et al., 2008), RelP and RelQ in *Streptococcus mutans* (Lemos *et al.*, 2007), and RelQ (EF2671) in *E. faecalis* (Abranches *et al.*, 2009) have been identified and characterized recently. These proteins were shown to harbour (p)ppGpp synthetase activity. Expression of the *ef2671* locus, encoding RelQ, was not modified in the $\Delta relA$ and $\Delta relA$ mutants (Table 3). To further determine a potential role of RelQ in (p)ppGpp production in the $\Delta relA$ mutants, we also constructed in this study a $\Delta relQ$ mutant and a $\Delta relA$ - $\Delta relQ$ double mutant, which gave (p)ppGpp accumulation profiles very similar to those of the wild-type V583 and $\Delta relA$ strains, respectively. This suggests a minor role for *relQ* in (p)ppGpp production under our experimental conditions.

To determine the role of the different (p)ppGpp production systems in the stringent response, we determined the effect of the antibiotic mupirocin, an inhibitor of isoleucyl-tRNA synthetase and a very efficient inducer of the stringent response in streptococci and enterococci (Lemos *et al.*, 2007; Abranches *et al.*, 2009). Results presented in Fig. 2(c) show that the addition of mupirocin induces a large (p)ppGpp accumulation in the strain V583, as well as in the $\Delta relA$ comp, $\Delta relA$ sp and $\Delta relQ$ strains, thus showing that *relA* is the main system involved in stringent response-dependent (p)ppGpp accumulation.

Collectively, our results show that the *ef1974* locus effectively encodes a (p)ppGpp synthetase, that it is the main system involved in the development of the stringent response and in (p)ppGpp production under our experimental conditions. As a consequence, we focused on determining the role of this system on stress adaptation and virulence of *E. faecalis*.

Effect of *relA* mutations on stress adaptation of *E. faecalis* V583

To determine whether RelA plays a central role in stress adaptation in *E. faecalis*, we first compared the ability of the parental strain V583 and isogenic mutants to grow in liquid GM17 medium under various stress conditions, i.e. 1 mM H_2O_2 , 1 M NaCl, 0.05 % bile salts and pH 5.5.

As shown in Fig. 3(a), no significant difference in the growth of the four strains occurred in the absence of stress. This phenotype resembles that observed for *L. lactis*, in which growth of the *relA** mutant in M17 was only barely reduced (Rallu *et al.*, 2000). In other phylogenetically related bacteria, e.g. *L. monocytogenes* (Okada *et al.*, 2002) and *S. mutans* (Lemos *et al.*, 2004), *relA* null mutants grew slower than their parental strains, but reached a final cell density equivalent to the parent at stationary phase.

Growth in media containing 1 mM H_2O_2 or 1 M NaCl revealed very contrasting phenotypes for the two mutants (Fig. 3b). Indeed, in media containing 1 mM H_2O_2 , the growth of the wild-type and the $\Delta relA$ strains was strongly inhibited [the doubling time (DT) increased over sevenfold to about 5.9 h, compared to 0.8 h for the three strains in

Fig. 2. Production of (p)ppGpp in *rel* mutants of *Enterococcus faecalis* V583. Cells were grown in GM17 to mid-exponential phase, and were transferred to DM (a) or GM17 (b, c) containing $150 \mu\text{Ci } ^{32}\text{P-labelled H}_3\text{PO}_4 \text{ ml}^{-1}$ for 2 h. In (c), the stringent response inducer mupirocin (Mupi) was added during the labelling process in the indicated lanes. After extraction, the labelled nucleotides were separated by PEI-cellulose TLC, and revealed using a Cyclone PhosphorImager (Packard).

Fig. 3. Effect of *relA* mutations on the growth of *E. faecalis* V583. Growth of strains V583 (●), $\Delta relA$ (■), $\Delta relAsp$ (▲) and $\Delta relAcomp$ (□) in GM17 (a), GM17 plus 1 mM H_2O_2 (b), GM17 plus 1 M NaCl (c), GM17 adjusted to pH 5.5 (d) or GM17 containing 0.05% bile salts (e). Results are the means of triplicates, with less than 10% error.

the absence of stress] (Fig. 3b). Interestingly, the $\Delta relAsp$ mutant was very tolerant to this stress, and displayed 4.5-fold faster growth than the wild-type or $\Delta relA$ strains under stress ($DT=1.3$ h). By contrast, in the presence of 1 M NaCl, the growth of the $\Delta relA$ mutant was reduced

($DT=2.2$ h), compared to that of strains V583 and $\Delta relAsp$ (which both display DT values near 1.5 h, Fig. 3c). The growth of the $\Delta relAcomp$ strain in all experiments was very similar to that of the wild-type strain. Only minor differences between the three strains were observed for growth in acidified GM17, and in media containing bile salts (Fig. 3d, e). As these growth experiments revealed clear phenotypes in NaCl- and H_2O_2 -stressed cultures, we determined the amount of (p)ppGpp in *E. faecalis* V583 and the various *rel* mutants under these conditions. The data presented in Fig. 4(a) show that the presence of NaCl has only a weak effect on (p)ppGpp production in V583 and the other strains. Considering these results, it would be tempting to speculate that the sensitivity of the $\Delta relA$ mutant results from its inability to produce (p)ppGpp. However, it should be noted that the $\Delta relA$ - $\Delta relQ$ double mutant – which is totally unable to produce (p)ppGpp – does not display any growth defect in high NaCl environments (data not shown and Abranches *et al.*, 2009). Results presented in Fig. 4(b) show that exposure to H_2O_2 triggered a decrease in the levels of (p)ppGpp. Considering the resistance of the $\Delta relAsp$ mutant to this stress, we hypothesized that this phenotype could be due to better – or at least maintenance of – (p)ppGpp production in the mutant under this type of stress. However, this hypothesis was not confirmed by the labelling experiments (Fig. 4b) that showed no difference between (p)ppGpp accumulation in the stressed V583 and $\Delta relAsp$ strains (Fig. 4b); thus the resistance of this mutant to oxidative stress appears to be independent of (p)ppGpp.

To test the ability of the two mutants to survive lethal challenges, cells were grown to exponential phase (same conditions as those preceding the labelling experiments showed in Fig. 2), and subjected to 0.3% bile salts, 25% ethanol, 45 mM H_2O_2 , pH 2.3 or a temperature of 60 °C.

Fig. 4. Production of (p)ppGpp during NaCl (a) and H_2O_2 (b) stresses in *E. faecalis* V583 and *rel* mutant derivatives. Cells were grown in GM17 plus 0.75 M NaCl (a) or GM17 plus 0.75 mM H_2O_2 (b) to mid-exponential phase, and were fed 150 μCi ^{32}P -labelled H_3PO_4 ml^{-1} for 2 h. Labelled nucleotides were separated by PEI-cellulose TLC, and revealed using a Cyclone PhosphorImager (Packard).

The challenge was conducted both in GM17 and after transfer of the cells into DM. Results presented in Fig. 5 revealed contrasting behaviours for the two mutant strains. Indeed, considering the experiments conducted in DM (Fig. 5a–e), the $\Delta relA$ strain appeared highly sensitive to H_2O_2 , but more resistant to ethanol, acid pH and bile salts than the wild-type strain. Although resistant to ethanol, acidity and to bile salts, the $\Delta relA$ mutant did not display any sensitivity to H_2O_2 . None of the mutants displayed a significant phenotype towards heat challenge (Fig. 5c). In the experiments conducted in GM17 (Fig. 5f–i), similar behaviours were observed when the mutants were subjected to heat, pH and bile salts. However, we did not observe any sensitivity of the $\Delta relA$ mutant when subjected to oxidative stress. In these survival experiments, the $\Delta relA$ strain always displayed the same behaviour as

the wild-type strain, thus allowing association of the phenotypes observed for the $\Delta relA$ mutant to the *relA* deletion.

E. faecalis is a natural resident of the animal gastrointestinal tract, and can be found in a wide variety of natural environments. As a consequence, during its life cycle, this bacterium is subjected to frequent changes of ecological niches, and displays a high degree on intrinsic resistance to environmental challenges (Flahaut *et al.*, 1997; Giard *et al.*, 2001). Large efforts have been made in recent years to understand the regulation of stress adaptation in *E. faecalis*. In this context, diverse transcriptional regulators such as PerR, HypR, Ers (Verneuil *et al.*, 2004, 2005; Giard *et al.*, 2006), several two-component systems (Le Breton *et al.*, 2003; Muller *et al.*, 2006, 2008), and extracytoplasmic

Fig. 5. Effect of *relA* mutations on the resistance of *E. faecalis* to lethal environmental stresses. Strains V583 (●), $\Delta relA$ (■), $\Delta relAmp$ (▲) and $\Delta relAcomp$ (□) were grown in DM (a–e) or in GM17 (f–i) to mid-exponential phase, and subjected to 25% ethanol (a), 45 mM H_2O_2 (b, f), 60 °C (c, g), pH 2.3 (d, h), or 0.3% bile salts (e, i).

function sigma factors (Benachour *et al.*, 2005) have been shown to be implied in adaptation to different stresses, but none of these fully explain the regulation of the general stress response in *E. faecalis* and, more generally, in Gram-positive bacteria lacking the σ^B homologue.

A few years ago, searching for acid-stress-resistant mutants in *L. lactis* MG1363, Rallu *et al.* (2000) discovered that mutants affected in their guanine nucleotides pools – in particular a *relA* mutant – displayed resistance to acid and multiple stresses, i.e. prolonged carbon starvation, oxidative stress and heat shock. This suggested a pivotal role for (p)ppGpp in the development of the general stress response in this bacterium. However, the position at which the transposon was inserted into the lactococcal *relA* gene allowed for the production of a 451 aa RelA protein. ^{32}P labelling showed that this mutant accumulated (p)ppGpp (Rallu *et al.*, 2000). It is noteworthy that another related RelA protein, the Rel_{Seq} protein from *Streptococcus equisimilis* also promotes (p)ppGpp accumulation when truncated to its 389 N-terminal amino acids (Mechold *et al.*, 2002). Collectively, these data suggested that a positive correlation between the level of stress resistance and the intracellular level of ppGpp should exist.

We designed our *ΔrelAsp* mutant to harbour a mutation similar to that occurring in the *Lactococcus* study (Rallu *et al.*, 2000). Thus we expected to (i) observe (p)ppGpp accumulation in this mutant, and (ii) obtain very contrasting phenotypes for the *ΔrelA* and *ΔrelAsp* mutants. The *ΔrelAsp* mutant, as expected, displayed a resistance phenotype towards several lethal stresses, i.e. acid pH, ethanol and bile salts, and grew better than the wild-type strain in the presence of H_2O_2 . However, none of the experimental conditions we tested allowed us to observe (p)ppGpp accumulation in this mutant, so the correlation between ppGpp accumulation and stress resistance does not appear to be so clear.

Conversely, *relA* null mutants are not uniformly sensitive to stresses. *relA* null mutants have been constructed in *S. mutans* and characterized (Lemos *et al.*, 2004). Interestingly, these mutants do not exhibit any phenotype when stressed with H_2O_2 and acidity in the planktonic state, but display increased resistance to acid challenge in a biofilm (Lemos *et al.*, 2004). In *L. monocytogenes*, a *relA* null mutant appears to be sensitive to osmotic stress (Okada *et al.*, 2002). In *E. faecalis* OG1RF, Abranches *et al.* (2009) showed that a *ΔrelA* mutant grew slower at 48 °C, at pH 5 and in the presence of 5% NaCl or 2 mM H_2O_2 . Consistently, we showed that the *ΔrelA* null mutant of *E. faecalis* V583 also appeared sensitive to hyperosmotic conditions and H_2O_2 (Fig. 3), but displayed increased resistance towards some lethal challenges (acid pH, ethanol and bile salts; Fig. 5). Interestingly, most of the stress phenotypes observed for the *ΔrelA* mutant in the OG1RF background (Abranches *et al.*, 2009) are reversed in a *ΔrelA-ΔrelQ* double mutant which is totally unable to produce (p)ppGpp (we also verified this for the NaCl stress

phenotype of our mutant). As a consequence, although our data argue for an important role of RelA in stress adaptation, it appears difficult at this time to strictly correlate the sensitivity or resistance to a given stress to the ability of the *E. faecalis* strains to produce (p)ppGpp, and the observed phenotypes most probably result from very fine tuning of the levels (p)ppGpp by RelA in stressed cells.

The *relAsp* mutant displays increased virulence in the *G. mellonella* infection model

The virulence of the *ΔrelA* and *ΔrelAsp* mutants was assayed using the *G. mellonella* infection model. In initial experiments, we aimed at determining the dose of V583 cells able to kill half of the insect population within 23 h. The dairy lactic acid bacterium *L. lactis* IL1403 was used as a non-pathogenic control. As shown in Fig. 6(a), the injection of *G. mellonella* with *E. faecalis* V583 resulted in the death of the caterpillars in a dose-dependent manner. Indeed, when 3×10^6 c.f.u. V583 was injected into the haemolymph of larvae, more than 90 % of the population was killed after 19 h. By contrast, with a fivefold lower dose (7×10^5 c.f.u.) or by injecting *L. lactis* (8×10^6 c.f.u.), no mortality was observed after 23 h.

With an injected dose of 1.5×10^6 c.f.u., about 33 % of the caterpillars still survived after 23 h. This dose was used in further experiments to determine whether the *relA* mutations affect virulence. The data presented in Fig. 6(b) show that the *ΔrelAsp* mutation dramatically increases the virulence of *E. faecalis* towards *G. mellonella*. Indeed, with the *ΔrelAsp* strain, almost 100 % of the caterpillars were dead after 21 h, whereas 60 % of the insect populations infected by the wild-type or the *ΔrelA* mutant strains were still alive. It is noteworthy that another mutant, carrying the same partial deletion as *ΔrelAsp*, but obtained independently, displayed similar phenotypes in both stress responses and virulence assays (data not shown).

For a number of human pathogens, a positive correlation between other infection models (e.g. virulence in mice, survival within macrophages) and virulence in *Galleria mellonella* has been found, establishing it as a useful model of infection (Jander *et al.*, 2000; Miyata *et al.*, 2003; Seed & Dennis, 2008). This model was used to show that the well known virulence factor GelE (gelatinase) of *E. faecalis* degrades an antimicrobial peptide and destroys the defence system in insect haemolymph, thus mimicking its role against the complement system in human serum (Park *et al.*, 2007).

In the present study, the *ΔrelAsp* mutant shows increased virulence towards *G. mellonella*, while the *ΔrelA* mutant did not display any phenotype. Many studies with Gram-negative pathogenic bacteria have shown a reduced virulence of stringent response mutants (mutated in *relA* or *spoT*, or both) unable to produce (p)ppGpp (Erickson *et al.*, 2004; Pizarro-Cerda & Tedin, 2004; Silva & Benitez, 2006). In Gram-positive bacteria, only a few mutational

Fig. 6. (a) Dose-dependent effect of *E. faecalis* V583 on the survival of the virulence model *Galleria mellonella*. Into *Galleria mellonella* 0.7×10^6 (○), 1.5×10^6 (□) and 3×10^6 (△) c.f.u. *E. faecalis* V583, and 8×10^6 c.f.u. *L. lactis* (◇) were injected, and the survival of the caterpillar population was followed for 23 h. (b) Effect of *relA* mutations on the virulence of *E. faecalis* towards *G. mellonella*. At time zero, 1.5×10^6 c.f.u. V583 (●), $\Delta relA$ (■) and $\Delta relAsp$ (▲) were injected.

studies have been performed. In their recent study, Abranches *et al.* (2009) did not observe any virulence phenotype for a $\Delta relA$ mutant (in the OG1RF genetic background) by using a *Caenorhabditis elegans* model. However, they showed that a $\Delta relA$ - $\Delta relQ$ double mutant was significantly attenuated [a phenotype we also observed in our $\Delta relA$ - $\Delta relQ$ mutant (data not shown), using our virulence model]. In *L. monocytogenes*, contradicting data are available. Using a mutant of *L. monocytogenes* EGD obtained by transposon insertional mutagenesis (position aa 162), Okada *et al.* (2002) did not observe any effect on virulence in a mouse infection model. By contrast, a negative effect on virulence of an insertional inactivation of the *relA* gene (position aa 211) was observed using the same murine model, but with strain C52 (Taylor *et al.*, 2002). Bennett *et al.* (2007) showed that a $\Delta relA$ mutation

alters intracellular growth within caco-2 cells. Taylor *et al.* (2002) also showed that *L. monocytogenes* mutants were impaired in their ability to grow after attachment on an inert surface. Similarly, $\Delta relA$ mutants of *S. mutans* reveal impaired ability to form a biofilm, a pivotal aspect of the pathogenic process of this bacterium (Lemos *et al.*, 2004). It should be noted that most *relA* mutants of Gram-positive bacteria described so far carry partial deletions of the *relA* gene. Considering the virulence phenotypes obtained with our two mutants, one being as virulent as the wild-type strain ($\Delta relA$), the other hypervirulent ($\Delta relAsp$), we have shown that different mutations in the same gene may lead to contrasting phenotypes that could explain the apparently contradictory results described for *Listeria*.

The expression of virulence of bacteria is intimately linked to their ability to adapt to stresses. During infection, pathogenic bacteria may encounter a variety of stresses within the host. Stresses may be encountered during passage through the gastro-intestinal tract (acid pH, bile salts), during internalization in macrophages (oxidative stress), or in the bladder (osmotic stress). As expected, a number of genes initially characterized as encoding effectors or regulators of the stress response of *E. faecalis* have been shown to be essential to its pathogenicity. As a consequence, if we compare the contrasting phenotypes observed for the two mutants and the wild-type strain, under stress conditions and in *Galleria*, we can correlate the increased virulence of the $\Delta relAsp$ mutant to its increased ability to proliferate in the presence of oxidative stress. Oxidative stress, through the release of superoxide by neutrophils in response to infection (i.e. the oxidative burst), is one of the key components of host defence. Interestingly, the innate immune responses of *Galleria* and of mammals share a number of structural and functional similarities. Among them, it has been shown that insect haemocytes do produce superoxide in response to infection (Bergin *et al.*, 2005).

In summary, by characterizing two mutants carrying different deletions in the *relA* gene, we have shown that RelA plays an important role in stress adaptation and virulence of *E. faecalis*. The two mutants displayed different phenotypes both in stress adaptation and in virulence, and the data in general suggest that the virulence of *E. faecalis* is linked to its ability to proliferate in an oxidative environment (rather than its ability to survive to oxidative challenge). An alternative hypothesis for this hypervirulence phenotype should be that the modified RelAsp protein may be toxic to *G. mellonella*. Analysis of the genes and proteins deregulated in mutants, by means of proteomics and transcriptomics, will undoubtedly provide interesting clues to better understand the regulation of stress adaptation and virulence in *E. faecalis*.

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